TITLE OF THE INVENTION:

MUTANT α-AMYLASES

BACKGROUND OF THE INVENTION

Field of the Invention:

The present invention relates to mutant liquefying alkaline α-amylases which have excellent heat resistance, and are particularly useful as enzymes for detergents, and genes thereof.

Description of the Background Art:

When an α-amylase [EC.3.2.1.1] is used as an enzyme for detergents, it has heretofore been said that a liquefying alkaline α-amylase, which can decompose starch at random and is stable to alkali and also to both chelating component and oxidation bleaching component, is preferred. However, in liquefying amylases, a calcium ion is generally important for maintaining the structure of the enzymes, and the stability thereof is lowered in the presence of a chelating agent. Besides, most of such enzymes have had the optimum pH in a neutral to weakly acidic range.

Under the foregoing circumstances, the present inventors found that enzymes produced by alkaliphilic *Bacillus* sp. KSM-K38 (FERM BP-6946) and *Bacillus* sp. KSM-K36 (FERM BP-6945) strains isolated from soil do not show the lowering of activity at all in the presence of a chelating agent at a high concentration by which

deactivation is recognized in the conventional liquefying α-amylases, and have resistance to surfactants and oxidizing agents and that they have higher activity on the alkaline side compared with the conventional liquefying α-amylases and are useful as enzymes for detergents (Japanese Patent Application No. 362487/1998.

However, said enzymes exhibit inactivation at a temperature of 50°C or higher, and so the heat resistance thereof have been somewhat insufficient in view of the fact that cleaning of clothing and tableware is generally conducted at about 10 to 60°C.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an α-amylase which is a liquefying alkaline α-amylase that has high activity on the alkaline side and is stable to both chelating component and oxidation bleaching component, and has excellent heat resistance.

The present inventors have acquired various mutant enzymes as to liquefying alkaline α-amylases and investigated them. As a result, it has been found that when a mutation is introduced into a specified amino acid residue in the amino acid sequence (SEQ ID NO:1) of amylase derived from KSM-K38, the heat resistance of the enzyme is improved without losing its properties such as resistance to chelating agents and resistance to oxidizing agents and high specific activity in an alkaline region, and that the heat resistance can be further improved by

combining such mutations.

According to the present invention, there is thus provided a mutant α-amylase obtained by making replacement or deletion of at least one residue of amino acid residues respectively corresponding to the 11th Tyr, 16th Glu, 49th Asn, 84th Glu, 144th Ser, 167th Gln, 169th Tyr, 178th Ala, 188th Glu, 190th Asn, 205th His and 209th Gln in the amino acid sequence set forth in SEQ ID NO:1 in an α-amylase having said amino acid sequence, or an α-amylase having a homology of at least 70% to said amino acid sequence.

According to the present invention, there is also provided a mutant α-amylase obtained by making replacement of a sequence corresponding to 11 to 100 amino acid residues from the amino terminal in the amino acid sequence set forth in SEQ ID NO:1 in an α-amylase having said amino acid sequence, or an α-amylase having a homology of at least 70% to said amino acid sequence by an amino acid sequence of another liquefying α-amylase corresponding to said sequence of the amino acid residues.

According to the present invention, there are further provided genes respectively encoding these mutant α-amylases, vectors having each of the genes, cells transformed by such a vector, and a production process of these mutant α-amylases, comprising culturing the transformed cells.

According to the present invention, there is still further provided a detergent composition comprising any

one of these mutant α-amylases.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and advantages of the present invention will become apparent from the following description and the appended claims, taken in conjunction with the accompanying drawings, in which:

Fig. 1 illustrates a method for preparing a recombinant plasmid for the production of α-amylases derived from KSM-K38 and KSM-AP1378 strains.

Fig. 2 illustrates a method for introducing a mutation into an α-amylase gene derived from the KSM-38 strain.

Fig. 3 illustrates a method for replacing an N-terminal sequence of the α-amylase gene derived from the KSM-38 strain by an N-terminal region of an α-amylase gene derived from the KSM-AP1378 strain.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The mutant α-amylases according to the present invention are obtained by mutating a gene encoding a liquefying alkaline α-amylase having the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having a homology of at least 70% to said amino acid sequence. However, an example where heat resistance is improved by deletion and/or replacement of an amino acid has also been conducted on the conventional

liquefying α-amylases. For example, an enzyme obtained by deleting residues from the 177th Arg to the 178th Gly in an enzyme derived from *B. amyloliquefaciens* (J. Biol. Chem., 264, 18933, 1989) and an enzyme obtained by replacing the 133rd His in an enzyme derived from *B. licheniformis* by Tyr (J. Biol. Chem., 265, 15481, 1990) have been reported. However, the liquefying alkaline α-amylases used in the present invention have a low degree of amino acid homology with the conventional liquefying alkaline α-amylases. In these α-amylases, a site corresponding to the residues from the 177th Arg to the 178th Gly has been already deleted, and the amino acid corresponding to the 133rd His has been already Tyr. Therefore, the examples of the conventional enzymes cannot be always applied. More specifically, the mutations of the amino acid sequence for improving the heat resistance in the present invention are entirely different from the examples up to the date.

Examples of the liquefying alkaline α-amylases include an enzyme (Japanese Patent Application No. 362487/1998) derived from a *Bacillus* sp. KSM-K38 (FERM BP-6946) strain separated from soil by the present inventors and having the amino acid sequence of SEQ ID NO:1 and an enzyme (SEQ ID NO:4) (Japanese Patent Application No. 362487/1998) derived from *Bacillus* sp. KSM-K36 (FERM BP-6945) and having a homology of about 95% to the amino acid sequence of SEQ ID NO:1. Incidentally, the homology of the

amino acid sequence is calculated in accordance with the Lipman-Pearson method (Science, 227, 1435, 1985).

In order to obtain the mutant α-amylase according to the present invention, a gene encoding a liquefying α-amylase is first cloned from microorganisms which produce said liquefying α-amylase. As a method therefor, a general gene recombination method may be used. For example, the method described in Japanese Patent Application Laid-Open No. 336392/1996 may be used. Examples of the gene include those set forth in SEQ ID NO:3 and SEQ ID NO:5.

A mutation is then introduced into the gene thus obtained. As a method therefor, any method may be adopted so far as it is a method of site-specific mutation commonly performed. The mutation can be performed, for example, by using a Site-Directed Mutagenesis System Mutan-Super Express Km kit produced by Takara Shuzo Co., Ltd. An optional sequence of the gene may be replaced by a sequence of another gene corresponding to the optional sequence by using the recombinant PCR (polymer chain reaction) method (PCR protocols, Academic Press, New York, 1990).

The mutation for improving the heat resistance in the present invention is desirably a mutation in which an amino acid residue corresponding to the 11th Tyr in the amino acid sequence set forth in SEQ ID NO:1 is replaced by Phe, an amino acid residue corresponding to the 16th Glu by Pro, an amino acid residue corresponding to the

49th Asn by Ser, an amino acid residue corresponding to the 84th Glu by Gln, an amino acid residue corresponding to the 144th Ser by Pro, an amino acid residue corresponding to the 167th Gln by Glu, an amino acid residue corresponding to the 169th Tyr by Lys, an amino acid residue corresponding to the 178th Ala by Gln, an amino acid residue corresponding to the 188th Glu by Asp, an amino acid residue corresponding to the 190th Asn by Phe, an amino acid residue corresponding to the 205th His by Arg, or an amino acid residue corresponding to the 209th Gln by Val.

The improvement of heat resistance can also be achieved by replacing an amino acid sequence corresponding to 11 to 100 amino acid residues from the amino terminal (Asp) in the amino acid sequence of SEQ ID NO:1 according to the present invention, preferably a sequence corresponding to amino acid residues from the 1st Asp to the 19th Gly, by an amino acid sequence of another liquefying α-amylase corresponding to said sequence of the amino acid residues.

Examples of said another liquefying α-amylase used in the replacement include an enzyme having the amino acid sequence set forth in SEQ ID NO:2. A site of its amino acid sequence corresponding to said amino acid residues from the 1st Asp to the 19th Gly is from the 1st His to the 21st Gly. The enzyme is an liquefied α-amylase derived from a *Bacillus* sp. KSM-AP1378 (FERM BP-3048)

strain, and the sequence of the gene is disclosed in Japanese Patent Application Laid-Open No. 336392/1996.

In the mutant α-amylases according to the present invention, a mutation with at least two kinds of replacement or deletion selected from the replacement or deletion of the above-described various kinds of amino acid residues and the replacement of the amino acid sequences combined with each other is also effective, and mutant enzymes more improved in heat resistance can be obtained by such a combination. More specifically, examples of the combination of mutations include a combination of at least two of the replacement or deletion of the various kinds of amino acid residues, a combination of at least two of the replacement of the amino acid sequence, and a combination of at least two of the replacement or deletion of the amino acid residues and the replacement of the amino acid sequence. Preferably, at least two mutations may be suitably combined from among mutations in which an amino acid residue corresponding to the 49th Asn is replaced by Ser, an amino acid residue corresponding to the 167th Gln by Glu, an amino acid residue corresponding to the 169th Tyr by Lys, an amino acid residue corresponding to the 190th Asn by Phe, an amino acid residue corresponding to the 205th His by Arg, and an amino acid residue corresponding to the 209th Gln by Val, and a mutation in which an amino acid sequence corresponding to amino acid residues from the 1st Asp to

the 19th Gly is replaced by an amino acid sequence from the 1st His to the 21st Gly in the amino acid sequence set forth in SEQ ID NO:2.

Examples of the most preferred combination include a combination of mutations in which an amino acid residue corresponding to the 49th Asn is replaced by Ser, an amino acid residue corresponding to the 167th Gln by Glu, an amino acid residue corresponding to the 169th Tyr by Lys, an amino acid residue corresponding to the 190th Asn by Phe, an amino acid residue corresponding to the 205th His by Arg, and an amino acid residue corresponding to the 209th Gln by Val, and a combination of a mutation in which an amino acid sequence corresponding to amino acid residues from the 1st Asp to the 19th Gly is replaced by an amino acid sequence from the 1st His to the 21st Gly in the amino acid sequence set forth in SEQ ID NO:2 with a mutation in which an amino acid residue corresponding to the an amino acid residue corresponding to the 167th Gln by Glu, an amino acid residue corresponding to the 190th Asn by Phe, or an amino acid residue corresponding to the 209th Gln by Val.

In addition, mutations for improving other properties than the heat resistance, for example, a mutation for more enhancing resistance to oxidizing agents, in which an amino acid residue corresponding to the 107th Met is replaced by Leu, a mutation for enhancing the detergency of a laundry detergent, in which an amino acid

residue corresponding to the 188th Glu is replaced by Ile, and/or the like may be combined with the above-described mutations.

The thus-obtained mutant α-amylases according to the present invention are improved in stability to heat without losing excellent properties of high resistance to chelating agents, and high specific activity in an alkaline region, and are hence useful for detergents for automatic dish washer, laundry detergents and desizing agents for fibers.

Such detergents may comprise one or more enzymes selected from debranching enzymes (for example, pullulanase, isoamylase, neopullulanase, etc.), α-glycosidases, glucoamylases, proteases, cellulases, lipases, pectinases, protopectinases, pectic acid lyases, peroxidases, laccases and catalases in addition to the above-described mutant α-amylases.

Further, surfactants such as anionic surfactants, amphoteric surfactants, nonionic surfactants and cationic surfactants, chelating agents, alkalizing agents, inorganic salts, resoiling preventives, chlorine scavengers, reducing agents, bleaching agents, fluorescent dye solubilizers, perfume bases, caking preventives, enzyme activators, antioxidants, preservatives, coloring matter, bluing agents, bleaching activators, enzyme stabilizers, phase adjusters, etc., which are commonly incorporated into the classical detergents, may be

incorporated.

The detergent composition according to the present invention can be produced by combining the above-described mutant α-amylases with the publicly known detergent components described above in accordance with a method known *per se* in the art. The form of the detergent composition may be suitably selected as necessary for the end application intended, and the detergent composition may be provided in the form of, for example, liquid, powder or granules. The detergent composition according to the present invention can be used as a laundry detergent, bleaching detergent, detergent for automatic dish washer, drain cleaner, artificial tooth cleaner or the like. In particular, it can preferably used as a laundry detergent, bleaching detergent or detergent for automatic dish washer.

The mutant α-amylases according to the present invention may be used as compositions for liquefaction and saccharification of starch and be also caused to act on starch together with one or more enzymes selected from glucoamylase, maltase, pullulanase, isoamylase, neopullulanase, etc.

The mutant α-amylases according to the present invention may also be used as desizing agent compositions for fibers by incorporating an enzyme such as pullulanase, isoamylase or neopullulanase.

EXAMPLES

Determination of amylase activity and protein content:

The amylase activity and protein content of each enzyme was determined in accordance with the following respective methods.

The determination of amylase activity was conducted by the 3,5-dinitrosalicylic acid method (DNS method). After a reaction was conducted at 50°C for 15 minutes in a reaction mixture with soluble starch contained in a 50 mM glycine buffer (pH: 10), reducing sugar formed was determined by the DNS method. With respect to the enzymatic activity, the amount of the enzyme, which forms reducing sugar corresponding to 1 μmol of glucose for 1 minute, was defined as 1 unit.

The protein content was determined by means of a Protein Assay Kit produced by Bio-Rad Laboratories making use of bovine serum albumin as a standard.

Referential Example 1:

Screening of liquefying alkaline amylase:

Soil (about 0.5 g) was suspended in sterilized water and subjected to a heat treatment at 80°C for 15 minutes. A supernatant of the heat-treated suspension was suitably diluted with sterilized water, and the resultant dilute solution was coated on an agar medium (Medium A) for separation. Culture was then conducted at 30°C for 2 days to form colonies. Those on the peripheries of which transparent halo based on amylolysis had been formed were screened, and isolated as amylase-producing bacteria.

Further, the thus-isolated bacteria were inoculated on Medium B and subjected to aerobic shaking culture at 30°C for 2 days. After the culture, the resistance performance to a chelating agent (EDTA) of a supernatant centrifugally separated was determined, and its optimum pH was further measured to screen the liquefying alkaline α-amylase-producing bacteria.

Bacillus sp. KSM-K38 (FERM BP-6946) and *Bacillus* sp. KSM-K36 (FERM BP-6945) strains were able to be obtained by the above-described process.

Medium A:	Trypton	1.5%
	Soyton	0.5%
	Sodium chloride	0.5%
	Colored starch	0.5%
	Agar	1.5%
	Na_2CO_3	0.5%
	(pH 10)	
Medium B:	Trypton	1.5%
	Soyton	0.5%
	Sodium chloride	0.5%
	Soluble starch	1.0%
	Na_2CO_3	0.5%
	(pH 10)	

The mycological natures of the KSM-K38 and KSM-K36 strains are shown in Table 1.

Table 1

	KSM-K36 strain	KSM-K38 strain
(a) Results of microscopic observation	Bacili having sizes of 1.0-1.2 μm x 2.4-5.4 μm for K36 stain and 1.0-1.2 μm x 1.8-3.8 μm for K38 strain. Oval endospores (1.0-1.2 μm x 1.2-1.4 μm) are formed at near end or the center thereof. Having periplasmic flagella and motility. Gram staining is positive. Having no acid-fast.	
(b) Growth state on various media: Incidentally, the strains are alkaliphilic and so 0.5% sodium carbonate was added to media used in the following tests.		
·Nutrient agar plate culture	Good growth state. Form of colonies is circular. Smooth surface and rough periphery. Color of colonies is pale-ocher.	Good growth state. Form of colonies is circular. Smooth surface and smooth periphery. Color of colonies is yellowish-brown.
·Nutrient agar slant culture	Grown.	Grown.
·Nutrient broth liquid culture	Grown.	Grown.
·Nutrient broth gelatin stab culture	Good growth state. No gelatin liquefaction is observed.	Good growth state. No gelatin liquefaction is observed.
·Litmus milk medium	Not changed.	Not changed.
(c) Physiological nature:		
·Reduction of nitrate and denitrification	Reduction of nitrate is positive. Denitrification is negative.	Reduction of nitrate is positive. Denitrification is negative.
·MR test	Failed to judge because the medium is alkaline.	Failed to judge because the medium is alkaline.
·V-P test	Negative.	Negative.
·Formation of indole	Negative.	Negative.
·Formation of hydrogen sulfide	Negative.	Negative.
·Hydrolysis of starch	Negative.	Negative.
·Citrate utilization	Grown on Christensen medium but not grown on Cocer and Simmons media.	Grown on Christensen medium but not grown on Cocer and Simmons media.
·Utilization of inorganic nitrogen source	Nitrate is utilized, but ammonium salt is not utilized.	Nitrate is utilized, but ammonium salt is not utilized.
·Formation of pigment	Formation of pale-yellow pigment on King B medium.	Negative.
·Urease	Negative	Negative
·Oxidase	Negative	Negative
·Catalase	Negative	Negative
·Range of growth	Temperature range for growth is 15-40°C, and optimum temperature range for growth is 30-37°C. pH range for growth is pH 8.0-11.0, and optimum pH for growth is pH 10.0-11.0.	Temperature range for growth is 15-40°C, and optimum temperature for growth is 30°C. pH range for growth is pH 9.0-11.0, and optimum pH for growth is the same as described above.
·Behavior against oxygen	Aerobic.	Aerobic.
·O-F test	Not grown.	Not grown.
·Sugar utilization	D-galactose, D-xylose, L-arabinose, lactose, glycerol, melibiose, ribose, D-glucose, D-mannose, maltose, sucrose, trehalose, D-mannit, starch, raffinose and D-fructose are utilized.	
·Growth on salt-containing medium	Grown at a salt concentration of 12%, but not grown at a concentration of 15%.	

Reference Example 2:

Culture of KSM-K38 and KSM-K36 strains:

The KSM-K38 or KSM-K36 strain was inoculated on the liquid medium B used in Referential Example 1 to conduct shaking culture at 30°C for 2 days. The amylase activity (at pH 8.5) of a supernatant centrifugally separated was determined. As a result, these strains had activities of 557 U and 1177 U per liter of the medium, respectively.

Referential Example 3:

Purification of liquefying alkaline amylase:

Ammonium sulfate was added to the resultant culture supernatant of the KSM-38 strain obtained in Referential Example 2 to 80% saturation. After stirring the resultant mixture, precipitate formed was collected and dissolved in 10 mM Tris-hydrochloride buffer (pH: 7.5) containing 2 mM $CaCl_2$ and dialyzed overnight against the same buffer. The dialyzate thus obtained was passed through a DEAE-Toyopearl 650M column equilibrated with the same buffer and caused to be adsorbed on the column, and the intended enzyme was eluted with the same buffer by 0-1 M gradient of sodium chloride concentration. After the active fraction was dialyzed against the same buffer, an active fraction obtained by gel filtration column chromatography was dialyzed against the above-described buffer, thereby obtaining a purified enzyme which gave a single band on both polyacrylamide gel electrophoresis (gel concentration: 10%) and sodium dodecyl sulfate (SDS)

electrophoresis. Incidentally, a purified enzyme was also able to be obtained from the culture supernatant of the KSM-K36 strain in accordance with the same process as described above.

Reference Example 4:

Properties of enzyme:

(1) Action:

Both enzymes decompose the α-1,4-glycoside bonds of starch, amylose, amylopectin and partially decomposed products thereof and produce glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7) from amylose. However, the enzymes do not act on pullulan.

(2) pH stability (Britton-Robinson's buffer):

Both enzymes exhibit a residual activity of at least 70% in a pH range of 6.5 to 11 under treatment conditions of 40°C and 30 minutes.

(3) Action temperature range and optimum action temperature:

Both enzymes act in a wide temperature range of 20 to 80°C and have an optimum action temperature of 50 to 60°C.

(4) Temperature stability:

Enzyme was incubated in a 50 mM glycine-sodium hydroxide buffer (pH: 10) at various temperature for 30 minutes and then residual anzymatic activity was measured. As a result, both enzymes showed a residual activity of at

least 80% at 40°C and a residual activity of about 60% even at 45°C.

(5) Molecular weight:

Both enzymes have a molecular weight of 55,000 ± 5,000 as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

(6) Isoelectric point:

Both enzymes have an isoelectric point of about 4.2 as measured by isoelectric focusing.

(7) Influence of surfactant:

Even when both enzymes are treated at pH 10 and 30°C for 30 minutes in a 0.1% solution of each of various surfactants such as sodium linear alkylbenzenesulfonates, sodium alkylsulfates, sodium polyoxyethylene alkylsulfates, sodium α-olefinsulfonates, the sodium salts of α-sulfonated fatty acid esters, sodium alkylsulfonates, SDS, soap and Softanol, they scarcely undergo inhibition of their activities (residual activity: at least 90%).

(8) Influence of metal salt:

Both enzymes were treated at pH 10 and 30°C for 30 minutes with each of various metal salts, thereby determining the influence thereof.

The K38 strain is inhibited by 1 mM Mn^{2+} (inhibitory rate: about 75%) and somewhat inhibited by both 1 mM Sr^{2+} and Cd^{2+} (inhibitory rate: 30 to 40%).

Example 1: Cloning of liquefying α-amylase gene

A chromosome DNA extracted from cells of the KSM-K38

strain in accordance with the method by Saito & Miura (Biochim. Biophys. Acta, 72, 619, 1961) was used as a template to amplify a gene fragment (about 1.5 kb) encoding a liquefying alkaline α-amylase (hereinafter referred to as "K38AMY") having an amino acid sequence set forth in SEQ ID NO:1 by PCR making use of primers K38US (SEQ ID NO: 19) and K38DH (SEQ ID NO: 20). The thus-amplified fragment was cleaved with a restriction enzyme *Sal* I, and then inserted into a *Sal* I-*Sma* I site of an expression vector pHSP64 (Japanese Patent Application Laid-Open No. 217781/1994), thereby preparing a recombinant plasmid pHSP-K38 with a structural gene of K38AMY bonded to a trailing end of a potent promoter derived from the alkaline cellulase gene of a *Bacillus* sp. KSM-64 (FERM P-10482) strain contained in pHSP64 (Fig. 1).

Similarly, a gene fragment (about 1.5 kb) encoding a liquefying alkaline α-amylase (hereinafter referred to as "LAMY") having an amino acid sequence set forth in SEQ ID NO:2, which had been obtained by using a chromosome DNA extracted from cells of a *Bacillus* sp. KSM-AP1378 (FERM BP-3048) strain (Japanese Patent Application Laid-Open No. 336392/1998) as a template, and amplified by PCR making use of primers LAUS (SEQ ID NO: 21) and LADH (SEQ ID NO: 22) was inserted into a *Sal* I-*Sma* I site of an expression vector pHSP64 in the same manner as described above, thereby preparing a recombinant plasmid pHSP-LAMY (Fig. 1).

Example 2: Preparation of mutant K38AMY gene-1

A Site-Directed Mutagenesis System Mutan-Super Express Km Kit produced by Takara Shuzo Co., Ltd. was used for a site-specific mutation. The recombinant plasmid pHSP-K38 obtained in Example 1 was first used as a template to conduct PCR making use of primers CLUBG (SEQ ID NO: 23) and K38DH (SEQ ID NO: 20), thereby amplifying a fragment of about 2.1 kb from the leading end of a potent promoter derived from the KSM-64 strain to the trailing end of the liquefying alkaline α-amylase gene. This fragment was inserted into a *Sma* I site of a plasmid vector pKF19k attached to the above kit to prepare a recombinant plasmid pKF19-K38 for introduction of mutation (Fig. 2).

After various kinds of oligonucleotide primers for introduction of site-specific mutation respectively set forth in SEQ ID NO:6 to NO:15 were 5'-phosphorylated with a T4 DNA kinase, each of the resultant products and pKF19-K38 were used to conduct a mutation-introducing reaction in accordance with a method described in the kit, and an *Escherichia coli* MV1184 strain (Competent Cell MV1184, product of Takara Shuzo Co., Ltd.) was transformed with the resultant reaction product. Recombinant plasmids were extracted from the resultant transformants to conduct base sequence analysis, thereby confirming the mutation.

The mutation-introduced gene was made a template plasmid upon introduction of a different mutation by inserting an expression promoter region and a mutant

K38AMY gene portion into the *Sma* I site of pKF19k again, thereby introducing another mutation in accordance with the same process as described above.

Each of the thus-obtained mutant recombinant plasmids was used as a template to conduct PCR making use of primers CLUBG (SEQ ID NO: 23) and K38DH (SEQ ID NO: 20), thereby amplifying each of mutant K38AMY gene fragments. This fragment was cleaved with a *Sal* I and then inserted into a *Sal* I-*Sma* I site of an expression vector pHSP64 (Japanese Patent Application Laid-Open No. 217781/1994) to prepare a plasmid for production of mutant K38AMY (Fig. 1).

Example 3: Preparation of mutant K38AMY gene-2 (chimera with LAMY gene)

Recombinant PCR was used for a mutation in which the N-terminal region of the K38AMY gene is replaced by its corresponding region of an LAMY gene (Fig. 3). The recombinant plasmid pHSP-K38 obtained in Example 1 was first used as a template to conduct PCR making use of primers K38DH (SEQ ID NO: 20) and LA-K38 (SEQ ID NO: 17), thereby amplifying a fragment encoding a sequence from the 20th Gln to C-terminal of the amino acid sequence of K38AMY set forth in SEQ ID NO: 1. On the other hand, the recombinant plasmid pHSP-LAMY was used as a template to conduct PCR making use of primers CLUBG (SEQ ID NO: 23) and LA-K38R (SEQ ID NO: 18), thereby amplifying a gene fragment encoding a sequence from the leading end of the potent promoter to the 21st Gly of the amino acid sequence

of LAMY set forth in SEQ ID NO: 2. Second PCR making use of both DMA fragments, and primers CLUBG (SEQ ID NO: 23) and K38DH (SEQ ID NO: 20) was conducted, thereby amplifying a gene fragment (about 2.1 kb) encoding a substituted mutant enzyme (hereinafter abbreviated as "LA-K38AMY") in which both fragments having respective complementary sequences derived from the primers LA-K38 (SEQ ID NO: 17) and LA-K38R (SEQ ID NO: 18) were bonded to the terminal, and a region encoding a sequence from the 1st His to the 21st Gly of LAMY and successively a region encoding a sequence from the 20th Gln to the C-terminal of K38AMY were bonded to the trailing end of the potent promoter. This gene fragment was cleaved with *Sal* I and inserted into a *Sal* I-*Sma* I site of an expression vector pHSP64 (Japanese Patent Application Laid-Open No. 217781/1994), thereby preparing a plasmid for production of mutant K38AMY (Fig. 1).

Example 4: Production of mutant liquefying alkaline α-amylase

Each of the various plasmids for production of mutant K38AMY obtained in Examples 2 and 3 was introduced into a *B*acillus *subtilis* ISW 1214 strain (*leu*A *met*B5 *hsd*M1) in accordance with the protoplast method (Mol. Gen. Genet., 168, 111, 1979) to culture the resultant recombinant *B*acillus *subtilis* at 30°C for 3 days in a liquid medium (containing 8% of corn steep liquor; 1% of meat extract; 0.02% of potassium primary phosphate; 5% of

maltose; 5 mM of calcium chloride; and 15 μg/mL of tetracycline). The resultant culture supernatant was dialyzed against a Tris-HCl buffer (pH: 7.0), and the dialyzate was caused to be adsorbed on a DEAE-Toyopearl 650M column equilibrated with the same buffer, and eluted by gradient of NaCl concentration. This eluate was dialyzed against a 10 mM glycine buffer (pH: 10.0), thereby obtaining a purified enzyme of each mutant K38AMY.

Example 5: Assay of heat resistance-1

Purified preparations of an enzyme (abbreviated as "Y11F") with the 11th Tyr in SEQ ID NO:1 replaced by Phe, an enzyme (abbreviated as "N49S") with the 49th Asn replaced by Ser, an enzyme (abbreviated as "E84Q") with the 84th Glu replaced by Gln, an enzyme (abbreviated as "S144P") with the 144th Ser replaced by Pro, an enzyme (abbreviated as "Q167E") with the 167th Gln replaced by Glu, an enzyme (abbreviated as "Y169K") with the 169th Tyr replaced by Lys, an enzyme (abbreviated as "A178Q") with the 178th Ala replaced by Gln, an enzyme (abbreviated as "E188D") with the 188th Glu replaced by Asp, an enzyme (abbreviated as "N190F") with the 190th Asn replaced by Phe, and an enzyme (abbreviated as "Q209V") with the 209th Gln replaced by Val were obtained in accordance with the processes described in Examples 1, 2 and 4, and their heat resistance was assayed by the following method. As a control, wild type K38AMY was used.

Each enzyme was added to a 50 mM glycine buffer (pH:

10.0) preincubated at 50°C so as to give a concentration of about 1.2 U/mL, and after 30 minutes, the buffer was sampled to determine the residual amylase activity of the enzyme in accordance with the method described above in EXAMPLES. The activity of the enzyme at the start is regarded as 100% to determine a relative activity, thereby regarding it as the residual amylase activity. The results are shown in Table 2. In the wild type K38AMY, the residual activity was decreased to 15%, while all the mutant enzymes exhibited a high residual activity compared with the wild type.

Table 2

Enzyme	Residual activity (%) after 30 minutes
Wild type	15
Y11F	40
N49S	30
E84Q	25
S144P	30
Q167E	46
Y169K	63
A178Q	20
E188D	30
N190F	70
Q209V	40

Example 6: Assay of heat resistance-2

Mutant enzymes with Q167E, Y169K, N190F and Q209V among the mutations described in Example 5 combined in the following manner were prepared in accordance with the processes described in Examples 1, 2 and 4.

Q167E/Y169K (abbreviated as "QEYK", prepared by using primer of SEQ ID NO: 16)

N190F/Q209V (abbreviated as "NFQV")

Q167E/Y169K/N190F/Q209V (abbreviated as "QEYK/NFQV")

With respect to these enzymes, the heat resistance was assayed by a method similar to Example 5. However, the temperature in the heat treatment was changed to 55°C, and Q167E, Y169K, N190F and Q209V were used as controls. As a result, as shown in Table 3, all the mutants were observed being improved in heat resistance by the combination, and QEYK/NFQV obtained by combining 4 mutations exhibited a residual activity of 85% after 30 minutes even at 55°C.

Table 3

Enzyme	Residual activity (%) after 30 minutes
Q167E	7
Y169K	14
QEYK	45
N190F	20
Q209V	1
NFQV	40
QEYK/NFQV	85

Example 7: Assay of heat resistance-3

The following mutant enzymes with the mutation NFQV described in Example 6 combined with S144P described in Example 5, and further combined with a mutation of replacement of 16th Gln by Pro (abbreviated as "E16P") were prepared in accordance with the processes described

in Examples 1, 2 and 4.

S144P/NFQV (abbreviated as "SP/NFQV")

E16P/S144P/NFQV (abbreviated as "EPSP/NFQV")

With respect to these enzymes, the heat resistance was assayed by a method (50°C) similar to Example 5. As a result, as shown in Table 4, improvement in heat resistance was observed by combining E16P with SP/NFQV.

Table 4

Enzyme	Residual activity (%) after 30 minutes
SP/NFQV	40
EPSP/NFQV	50

Example 8: Assay of heat resistance-4

The following mutant enzymes with QEYK/NFQV among the mutations described in Example 6 suitably combined with a mutation (abbreviated as "M107L") with the 107th Met in SEQ ID NO:1 replaced by Leu, a mutation (abbreviated as "H205R") with the 205th His replaced by Arg, and N49S among the mutations described in Example 5 were prepared in accordance with the processes described in Examples 1, 2 and 4.

M107L/QEYK/NFQV (abbreviated as "ML/QEYK/NFQV")

N49S/M107L/QEYK/NFQV (abbreviated as "NSML/QEYK/NFQV")

N49S/M107L/H205R/QEYK/NFQV (abbreviated as "NSMLHR/QEYK/NFQV")

With respect to these enzymes, the heat resistance was assayed by a method similar to Example 5. However, the temperature in the heat treatment was changed to 60°C.

As a result, heat resistance was additionally improved by combining ML/QEYK/NFQV with N49S, further H205R, and NSMLHR/QEYK/NFQV exhibited a residual activity of 75% after 30 minutes even at 60°C (Table 5)

Table 5

Enzyme	Residual activity (%) after 30 minutes
ML/QEYK/NFQV	30
NSML/QEYK/NFQV	50
NSMLHR/QEYK/NFQV	75

Example 9: Assay of heat resistance-5

A mutant enzyme LA-K38AMY with a sequence from the 1st Asp to the 19th Gly of K38AMY replaced by a sequence from the 1st His to the 21st Gly of LAMY was obtained in accordance with the processes described in Examples 1, 3 and 4. The heat resistance of this enzyme was assayed by the method described in Example 5. As a result, as shown in Table 6, improvement in heat resistance by the replacement was observed.

Table 6

Enzyme	Residual activity (%) after 30 minutes
Wild type	15
LA-K38AMY	33

Example 10: Assay of heat resistance-6

Into the gene of the mutant enzyme QEYK/NFQV described in Example 6, was introduced a mutation with a sequence from the 1st Asp to the 19th Gly replaced by a sequence from the 1st His to the 21st Gly of LAMY in accordance with the same processes as in Examples 1 and 3. With respect to a mutant enzyme LA-K38AMY/QEYK/NFQV obtained by using this enzyme in accordance with the process described in Example 4, the heat resistance was assayed by the same method (heat treatment temperature: 60°C) as in Example 8.

As a result, heat resistance was additionally improved by the combination, and LA-K38AMY/QEYK/NFQV exhibited a residual activity of 63% after 30 minutes even at 60°C (Table 7)

Table 7

Enzyme	Residual activity (%) after 30 minutes
LA-K38AMY	1
QEYK/NFQV	40
LA-K38AMY/QEYK/NFQV	63

Example 11: Detergent composition for automatic dish washer

A detergent composition for automatic dish washer was produced in accordance with a formulation shown in Table 8, and various mutant enzymes were separately incorporated into this detergent composition to conduct a

washing test. As a result, the mutant enzymes exhibited an excellent detergent effect compared with the wild type enzyme when the enzymes having the same activity value as each other are added.

Table 8

Composition of detergent	(%)
Pluronic L-61	2.2
Sodium carbonate	24.7
Sodium hydrogencarbonate	24.7
Sodium percarbonate	10.0
Sodium silicate No. 1	12.0
Trisodium citrate	20.0
Polypropylene glycol	2.2
Silicone KST-04 (product of Toshiba silicone Co., Ltd.)	0.2
Socarane CP-A45 (product of BASF AG)	4.0

The mutant α-amylases according to the present invention have excellent properties of high resistance to chelating agents, high specific activity in an alkaline region, excellent stability to heat, and are hence useful for detergents for automatic dish washer, laundry detergents, compositions for liquefaction and saccharification of starch, and desizing agents for fibers.